2.0 INTRODUCTION

This study was conducted to demonstrate the validity of Analytical Method GRM042.02A (Reference 1) for determination of residues of SYN545192 in soil.

The objectives of this study are:

a) To establish that the method produces acceptable recovery values for SYN545192, i.e. recoveries are in an expected range between 70% and 110% with relative standard deviation (≤ 20%) in soil;

b) To establish that the limit of quantitation (LOQ) of the analytical method is 0.001 mg/mL (1 ppb) for SYN545192 in soil;

c) To establish that the residues of SYN545192 or interferences in control samples or reagent blanks are not present at levels above 30% of the LOQ;

d) To demonstrate that the relationship between the instrument (LC-MS/MS) response and amount of SYN545192 injected on column is linear over the working range of the method;

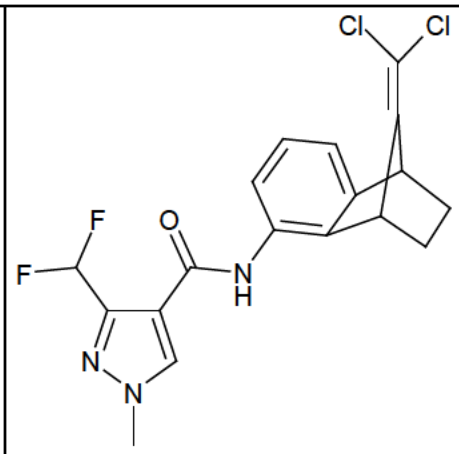
e) To assess any effect of the presence of matrix on the instrument response;

f) To assess the stability of SYN545192 in soil sample extracts and in final solutions;

g) This study has been designed to comply with OECD Guidance Document ENV/JM/MONO(2007)17, EPA guidelines OPPTS 860.1340, EPA Guidelines OPPTS 850.7100, EU Guidance SANCO/3029/99 Rev. 4 and SANCO/825/00 Rev. 7.

3.0 MATERIALS AND METHODS

3.1 Test substance

Compound	
Common Name:	Hambra
Code Name:	SYN545192
IUPAC Name:	3-Difluoromethyl-1-methyl-1H-pyrazole-4-carboxylic acid (9-dichloromethen-1,2,3,4-tetrahydro-1,4-methano-naphthalen-5-yl)-amide
CAS Number:	Not in registry
Molecular Formula:	$C_{18}H_{15}Cl_2F_2N_3O$
Molecular Weight:	398
Source:	Syngenta Product Safety Greensboro---Logistics and Support
Standard Reference:	DAH-XXXIII-76
Purity:	98.4%
Storage Conditions:	Refrigerated
Expiration Date:	5/31/2011

3.2 Test System

This validation study was carried out using two types of untreated soil samples transferred from previously conducted Syngenta Study T004880-06 (clay loam) (Reference 2) and Study T004878-06 (sandy loam) (Reference 3). These control soil samples were characterized by Agvise Laboratories of Northwood, North Dakota and reported to Syngenta on May 24, 2007 for Syngenta Study T004880-06 (clay loam) and T004878-06 (sandy loam). The GLP characterization results of the control soils are summarized in Table 1.

3.3 Preparation of Analytical Standard Solutions

The stock solution of SYN545192 was prepared at 100 µg/mL concentration in acetonitrile for this study. For preparing the fortification standards, the stock solution was diluted with acetonitrile to 0.1 µg/mL and 0.01 µg/mL, respectively. Further dilutions were made with

50/50 H_2O/acetonitrile to yield calibration standards at 0.02 ng/mL, 0.05 ng/mL, 0.1 ng/mL, 0.2 ng/mL, 0.5 ng/mL and 1 ng/mL.

3.4 Fortification Levels

Procedural recoveries of SYN545192 through the analytical procedures were assessed by fortifying untreated soil samples with the appropriate fortification standard solutions (Section 3.3). The volumes of the fortification standard added were 1.0 mL. For each type of the soils, five recovery samples were prepared at the proposed LOQ, i.e. 1 ppb, and five recovery samples were prepared at 10X LOQ, i.e. 10 ppb. In addition, two untreated control samples were analyzed for each type of the soils. The fortification levels are summarized in Table 2.

3.5 Analytical Procedures

3.5.1 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limit of detection (LOD) of the method is defined as the lowest analyte concentration detectable above the mean amplitude of the background noise in an untreated sample at the corresponding retention time. An estimate of the LOD can be taken as three times background noise and injections of 1 pg of SYN545192 were reliably quantified with a signal to noise ratio of significantly greater than 3:1. The limit of quantitation (LOQ) was determined for this method by analyzing the recovery samples fortified at 1 ppb for SYN545192.

3.5.2 Sample Analysis

For each type of the soil tested, a total of twelve subsamples of soil (10 grams) were weighed into round bottom flasks (100 mL) as a set. Five of the samples were spiked with SYN545192 at 1 ppb (0.001 mg/kg) and five were at 10 ppb (0.01 mg/kg). Two of the samples remained untreated as controls. The samples were refluxed each with 50 mL of 80/20 acetonitrile/H_2O (v/v) for 60 minutes. After the sample mixtures were cooled to room temperature, aliquots (2.5 mL) were taken from the extracts and diluted to 25 mL with H_2O. These samples were passed through pre-conditioned Waters HLB SPE cartridges (60 mg, 3 mL) and rinsed each with 2 mL of 90/10 (v/v) H_2O/acetonitrile. The residues of SYN545192 were eluted from the SPE cartridges each with 2 mL of acetonitrile. The final volumes were adjusted with H_2O to 4 mL for control and recovery samples at 1 ppb. For recovery samples fortified at 10 ppb, the final volumes were adjusted with H_2O to 4 mL and then further diluted to 20 mL with 50/50 (v/v) H_2O/acetonitrile. Aliquots (~1 mL) were transferred from the sample final solutions to 2 mL injection vials for LC-MS-MS analysis.

3.5.3 LC-MS/MS Instrumentation

For analysis of SYN545192, an Applied BioSystem API 4000 triple-quad mass spectrometer was optimized and operated under negative mode TurboSpray ionization with a negative MRM transition *m/z* 396→368 as primary and a second negative MRM transition *m/z* 396→91 as confirmatory. Two Perkin 200 LC pumps and an Agilent SB AQ column (50

mm x 4.6 mm i.d., 3.5 µm particle size) were used for LC separations. The LC operation parameters are summarized as below:

Mobile Phase A:	0.2% acetic acid in H_2O
Mobile Phase B:	Acetonitrile
Flow Rate:	1 mL/min
Column Oven Temp:	40°C
Injection Vol.	50 µL
Run Time:	5 minutes

Time (Min)	A% (0.2% acetic acid)	B% (Acetonitrile)	Flow (mL/min)	Gradient curve
0.0	80	20	1	
3.0	10	90	1	Linear
4.0	10	90	1	
4.1	80	20	1	Linear
5.0	80	20	1	

MS/MS Conditions:

Source Temperature:	450 °C
CAD Gas:	8
Curtain Gas:	40
GS1:	50
GS2:	50
IS:	-3500
Q1 Resolution:	Unit
Q3 Resolution:	Unit
Dwell time:	150 ms

Analyte	DP	EP	CE	CXP
SYN545192	-75	-10	-22	-11
SYN545192 (confirmatory)	-75	-10	-60	-5

Data Acquisition: Raw area counts are downloaded from the Analyst 2 data collection system (Version 1.4.2) to Syngenta SAW spreadsheet to calculate the final results.

3.5.4 Sample Calculations

Peak integration, peak area count quantitation and development of calibration curves were performed by Analyst 2 (version 1.4.2) associated with LC-MS/MS instrument. Analyte weight (pg) injected on column for each sample was calculated per the calibration curve (y = mc + b). The residue concentrations (ppb) were calculated according to the equations below:

$$\text{Residues (ppb)} = \frac{\text{analyte injected (pg) on column}}{\text{sample matrix injected on column (mg)}}$$

$$\text{Matrix injected (mg)} = \left(\frac{\text{sample wt (g)}}{\text{extract vol} * \text{ (mL)}} \right) \times \left(\frac{\text{aliquot vol (mL) x inj vol (uL)}}{\text{final volume (mL)}} \right)$$

$$\text{Extract volume} * = \text{extraction solvent volume (mL)} + \text{sample weight (g)} \times \text{moisture(\%)}$$

$$\text{Recovery} = \frac{\text{ppb found in recovery sample - ppb found in control}}{\text{ppb fortified}}$$

The calculations of residue concentrations and recoveries were performed using a Syngenta Saw Excel Spreadsheet. A representative Excel spreadsheet is presented in Appendix 1 for calculation verification. A statistical treatment of the data includes the calculation of averages, standard deviations, relative standard deviations. Mean percent recoveries, standard deviations, and relative standard deviations were calculated using Microsoft® Office Excel 2003. Results were rounded for reporting purposes but not during calculations.

4.5 Matrix Effects

In order to observe the matrix effects for the method, an experiment was conducted as follows: For each of the soil types, matrix matched standards were prepared in triplicate from untreated samples (control) taken through the analytical procedures and the final solutions (4 mL) were spiked each with 40 µL of the 0.01 µg/mL fortification standard of SYN545192. In the meantime, two freshly prepared 0.1 ng/mL non-matrix standards were prepared by spiking 40 µL of the 0.01 µg/mL fortification standard of SYN545192 in 4 mL of final solvent (50/50 (v/v) H_2O/acetonitrile). These samples were injected into the LC-MS/MS system and analyzed by both primary and confirmatory transitions. The matrix effects were calculated as follows:

$$\text{Matrix Effects} = \frac{\text{peak area of standard in matrix - peak area of standard in solvent}}{\text{peak area of standard in solvent}} x100\%$$

It is found that the matrix effects, determined by primary transition (negative MRM *m/z* 396→368), were -11% and -15% for clay loam and sandy loam, respectively. The matrix effects were -10% and -25% for clay loam and sandy loam by confirmatory transition (negative MRM *m/z* 396→91). These results are summarized in Table 4.